Calcium: an insignificant thing

Christian Frøkjær-Jensen & Erik M Jorgensen

Fusion of synaptic vesicles upon calcium influx requires precise localization of voltage-gated calcium channels. A new study identifies a previously uncharacterized protein that mediates trafficking of Ca_V2 calcium channels in *C. elegans*.

In the presynaptic terminal, a puff of calcium is an insignificant thing, a scintilla painted on the dark ceiling of the synaptic bouton. First visualized in 1992 in the synaptic terminals of the squid, these intracellular calcium increases are transient and highly local, confined to microdomains[1]. The portals for extracellular calcium are voltage-gated calcium channels, usually of the Ca_V2 class, clustered at the active zone. Depolarization of the membrane opens the pore and a surge of calcium, reaching concentrations of 100 μM, flows into the cell[2]. However, this rise in calcium probably only extends 20 nm or so before dissipating; calcium diffusion is limited by the action of internal buffers that are very fast acting[3,4]. The calcium sensor involved in the fusion of synaptic vesicles with the membrane has a low affinity for calcium; it requires every bit of that 100 μM for effective release of neurotransmitter[5]. If the calcium channel is not near the synaptic vesicle, then there will be no neurotransmission. So, where are the channels? Who docks them there? Who pilots the tug? There must be escorts that regulate the synthesis, transport and localization of voltage-gated calcium channels to these sites. In this issue, Saheki and Bargmann[6] labeled the calcium channels with green fluorescent protein (GFP) and localized them to nematode synapses. They then used a simple *in vivo* visual genetic screen to identify the proteins that were required to transport and localize calcium channels to presynaptic sites in *C. elegans* and proposed a mechanism of calcium-channel trafficking.

There is a long and difficult history for studies of calcium-channel localization. For example, one study used a combination of electrophysiology and electron microscopy[2], finding that there are approximately 1,800 calcium channels in 20 discrete clusters on isolated hair cells. The study also estimated an almost identical number of ion channels on the basis of freeze-fracture electron microscopy and serial-section transmission electron microscopy; from these results, the authors concluded that calcium channels are positioned within 100 nm of the presynaptic active zone. However, these are very difficult experiments and, in the end, indirect. For studies of the mechanism and dynamics of Ca_V channel trafficking, it would be nice to just be able to see the channels directly in living cells.

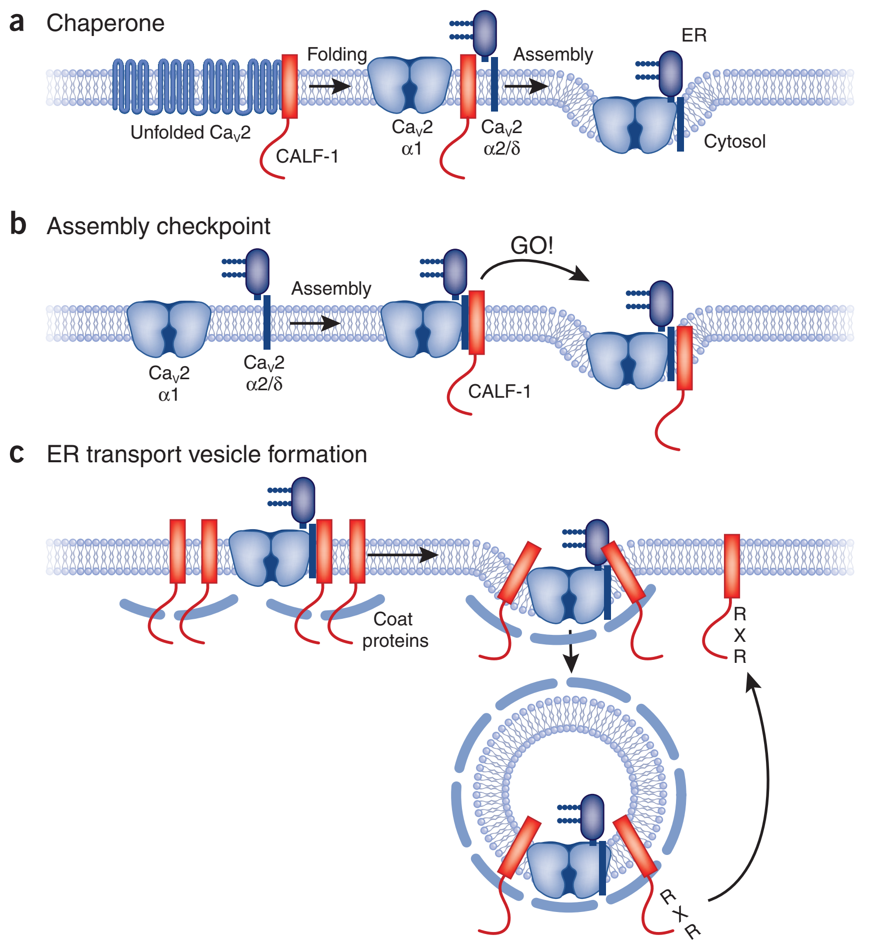

Figure 1 Ca_V2 calcium channel trafficking in *C.elegans*. Saheki and Bargmann[6] identified two proteins that are necessary for Ca_V2 channel transport and localization to presynaptic sites: CALF-1 and the $\alpha_2\delta$ subunit UNC-36. Ca_V2 channels are retained in the endoplasmic reticulum (ER) in the absence of either CALF-1 or UNC-36. There are at least three possible models for the function of CALF-1. (**a**) CALF-1 permanently resides in the endoplasmatic reticulum and acts as a specific chaperone for Ca_V2. Chaperone functions include channel folding and subunit assembly. (**b**) CALF-1 serves as an endoplasmatic reticulum checkpoint that monitors the assembly of Ca_V2 channels. At the checkpoint, only assembled channels are allowed to exit the endoplasmatic reticulum. Although not shown here, it is possible that a fully assembled Ca_V2 channel occludes the RXR motif and that a CALF-1/Ca_V2 complex is incorporated into the transport vesicle. (**c**) CALF-1 stimulates Ca_V2 channel export from the endoplasmatic reticulum by concentrating Ca_V2 channels at endoplasmatic reticulum export sites and by recruiting coat proteins necessary to form transport vesicles. In this model, the main function of the RXR motif is to retrieve CALF-1 to the endoplasmatic reticulum.

The authors are in the Department of Biology, University of Utah, Howard Hughes Medical Institute, Salt Lake City, Utah. Christian Frøkjær-Jensen is also at the Danish National Research Foundation Center for Cardiac Arrhythmia, Department of Biomedical Sciences, University of Copenhagen, Denmark.
e-mail: jorgensen@biology.utah.edu

To visualize calcium channel localization, Saheki and Bargmann[6] tagged a functional Ca_V2 channel with GFP and expressed it in a pair of neurons that make synapses along their axons in stereotyped positions. In these experiments, the tagged calcium channel specifically localizes to presynaptic active zones. Notably, this pattern can be observed in living worms by epifluorescence. Using this, the authors were able to screen for mutants in which Ca_V2 channel localization is disrupted. It is not a particularly easy screen, as worm screens go; it requires that every worm be mounted on a fluorescence microscope and scored for mislocalization. Nevertheless, hard screens can pay off, and the authors isolated mutants with mislocalized Ca_V2 channels and identified two proteins that are necessary for correct Ca_V2 transport: a previously unknown protein, calcium channel localization factor 1 (CALF-1), and an $\alpha_2\delta$ subunit.

CALF-1 is a small protein, composed of a single transmembrane domain and a cytosolic tail, that resides in the endoplasmatic reticulum. Saheki and Bargmann[6] found that the primary function of CALF-1 is in calcium-channel biogenesis; in the absence of CALF-1, Ca_V2 channels were retained in the endoplasmatic reticulum, whereas other active zone and synaptic vesicle components were properly localized. Endoplasmatic reticulum retention is not a developmental defect, as expression of CALF-1 in *calf-1* mutant adults promoted rapid exit of functional Ca_V2 channels from the endoplasmatic reticulum and transport to synaptic sites. How does CALF-1 promote Ca_V2 exit from the endoplasmatic reticulum? For most ion channels, endoplasmatic reticulum retention motifs are contained in the channels themselves. After channel assembly and maturation, outfitter proteins mask the retention signal and allow channels to exit the endoplasmatic reticulum[7]. In this case, however, it is not the Ca_V2 channel itself, but the accessory protein CALF-1, that has the endoplasmatic reticulum retention motif; the cytosolic tail of CALF-1 contains multiple arginine-x-arginine (RXR) endoplasmatic reticulum retention motifs embedded in basic and proline-rich regions.

In their genetic screen, Saheki and Bargmann[6] also isolated new mutant alleles of the $\alpha_2\delta$ subunit UNC-36. The $\alpha_2\delta$ subunit appears to have related Ca_V2 trafficking functions to CALF-1. $\alpha_2\delta$ subunits are accessory subunits to Ca_V channels that, in mammalian systems at least, increase the number of functional Ca_V channels in the cell membrane[7]. $\alpha_2\delta$ subunits are mainly extracellular, with the α_2 subunits being tethered to the extracellular face of the membrane by the δ subunit. *unc-36* mutants are uncoordinated, similar to Ca_V2 mutants, and GFP-tagged Ca_V2 is no longer detectable at presynaptic sites.

Is UNC-36 mainly involved in trafficking or does it also have a functional role? One experiment in particular demonstrated that $\alpha_2\delta$ has a functional role in nematodes. In $\alpha_2\delta$ mutants, overexpression of the CALF-1 protein partially restored Ca_V2 channel localization to synapses. However, locomotion was not restored, arguing for a role of $\alpha_2\delta$ in both channel function and trafficking. These results are consistent with data from mammalian and *Drosophila* studies, although the effects in *C. elegans* are more severe. In mammalian cell culture, $\alpha_2\delta$ promotes Ca_V channel surface expression and alters subtle functional properties of calcium currents[7]. In flies, the $\alpha_2\delta$ mutant *straightjacket* has reduced neuronal transmission as a result of a reduction in Ca_V2 channels at the synapse[8,9]. These studies underscore an important point, that $\alpha_2\delta$ proteins are bona fide subunits of the calcium channel complex and assembly of these subunits is likely to be permissive for trafficking, whereas CALF-1 is more likely to be specifically involved in trafficking the complex.

From these results, Saheki and Bargmann[6] propose that the $\alpha_2\delta$ subunits and CALF-1 promote exit from the endoplasmatic reticulum. As an underlying mechanism, three possible processes come to mind: folding, a checkpoint for assembly and formation of transport vesicles (**Fig. 1**)[10]. In the first possibility, CALF-1 functions as a chaperone for protein folding or promotes assembly of the subunits of the calcium channel complex. Failure to assemble the complex blocks these proteins from exiting the endoplasmatic reticulum. In the second possibility, CALF-1 functions as a checkpoint protein, similar to a licensing factor, that allows the fully assembled complex to exit. In the third possibility, CALF-1 interacts with the calcium channel at the endoplasmatic reticulum exit site for the formation of transport vesicles – for example in the recruitment of coat proteins. The authors do not favor a particular mechanism, but they exclude the possibility that the endoplasmatic reticulum retention motif of CALF-1 acts as a specific brake for an unassembled complex. First, loss of CALF-1 or elimination of the endoplasmatic reticulum retention motif did not lead to constitutive exit of the calcium channel. Second, substitution of the cytosolic tail of CALF-1 with the endoplasmatic reticulum retention motif from the adrenergic receptor partially rescued channel trafficking. Thus, the endoplasmatic reticulum retention motif probably functions to return CALF-1 to the endoplasmatic reticulum rather than being directly involved in calcium channel trafficking. Although CALF-1 does not have any obvious homologs outside of nematodes, the authors noted that gamma subunits of Ca_V channels in mammals share similarities, such as the RXR motifs and a proline-rich region, with CALF-1. It will be interesting to determine whether mammalian gamma subunits have similar roles in the biogenesis of Ca_V channels.

Saheki and Bargmann's study brings a number of questions to mind. For example, how do neurons regulate the number of Ca_V2 channels at synapses? At mammalian synapses, it has been proposed that there are a certain number of 'slots' for each type of Ca_V2 channel[11]. In Saheki and Bargmann's study[6], calcium channels at individual synapses are visible under conventional fluorescence microscope. Such a bright signal suggests that there are a substantial number of channels per synapse; however, not all of the tagged channels are necessarily inserted into the membrane. Previous experiments suggest that there may be very few calcium channels at synapses in *C. elegans*. It has been estimated that there are less than two Ca_V channels per synapse at one type of sensory neuron[12]. If quantitative studies bear these numbers out, calcium channels really do look like an insignificant component of the active zone, at least numerically speaking. However, Napoleon once said, "There are times when the most insignificant thing can decide the outcome of a battle." It is possible that the placement of just a single channel determines whether a particular synapse fires or remains silent. The tiny puff of calcium from a channel is not to be dismissed lightly, as all of neurotransmission hinges on its function. We are now closer to understanding how that speck positioned itself to become so important.

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